Rejection under 35 U.S.C. § 101

Claims 12-25 are rejected under 35 U.S.C. § 101 for lack of utility. The Examiner states that the claimed invention is not supported by a specific asserted utility or a well established utility. Applicants respectfully disagree.

Under M.P.E.P § 706.03(a)(1) the specification and claims are to be reviewed to determine if the applicant has asserted any credible utility for the claimed invention. Specifically, section 706.03(a)(1)(B)(1) states:

> If the applicant has asserted that the claimed invention is useful for a particular purpose (i.e., a "specific utility") and that assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility. Credibility is to be assessed from the perspective of one of ordinary skill in the art in view of any evidence of record (e.g., data, statements, opinions, references, etc.) that is relevant to the applicants assertions. An applicant must provide only one credible assertion of specific utility for any claimed invention to satisfy the utility requirement.

Applicants submit that the claimed invention has a wide variety of utilities, including the creation of DNA diagnostic probes labeled with electron transfer moieties. The claimed invention, nucleosides comprising electron transfer moieties, are used to make the labeled probes of the invention. These probes possess unique structural features, i.e., the capability to transfer electrons over large distances at fast rates, which enable them to be used as bioconductors and diagnostic probes. See specification at page 6, lines 20-23. Thus, the claimed invention directed toward compositions comprising nucleosides modified with electron transfer moieties has a specific asserted utility.

The Examiner states that the claimed invention lacks a well established utility because a person of skill in the art would not expect that a nucleotide with an attached electron transfer moiety would be incorporated during the synthesis of an oligonucleotide because the

bulky structure of the electron transfer moiety would interfere with the synthesis. Applicants respectfully disagree.

The specification describes a variety of methods to incorporate nucleosides and nucleotides modified with ETMs into nucleic acids. For example, methods for adding electron transfer moieties to modified nucleotides located at the 3', 5' termini, or an internal base of an oligonucleotide are described in the specification beginning at page 19, line 31, through page 25, line 10. Example 1 describes the synthesis of an oligonucleotide duplex with ETMs at the 5' termini (specification at page 37, line 21, through page 39, line 17); Example 2 describes the synthesis of long DNA duplexes with ETMs (page39, line 18 through page 40, line 6); Example 3 describes the synthesis of covalently bound ETMs at internucleotide linkages of duplex DNA (page 40, line 7 through page 41, line 4); Example 4 describes the synthesis of two oligonucleotides, each with an ETM attached at the 5' terminus (page 41, lines 5-19); Example 5 describes the characterization of modified nucleic acids made according to the method described in Example 1 (page 41, line 20 through page 43, line 3); and, Example 6 describes the synthesis of a single stranded nucleic acid labeled with two ETMs (page 43, line 4 through page 44, line 2).

Accordingly, applicants submit that the present invention directed towards compositions comprising modified nucleosides has both a specific asserted utility and a well established utility. Withdrawal of the rejection of claims 12-25 under § 101 is respectfully requested.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 12-25 are rejected under 35 U.S.C. § 112, first paragraph, because the claimed invention is not supported by a specific asserted utility or a well established utility. As

argued above, applicants have identified a specific utility for the claimed invention. In addition, as required by M.P.E.P § 706.03(a)(1) (b)(3), applicants have indicated where support for the asserted utility can be found in the specification.

Claims 12-25 are rejected under 35 U.S.C. § 112, first paragraph, for lack of written description. Specifically, the office action states that the specification fails to describe the claimed "nucleoside comprising a covalently attached electron transfer moiety".

Applicants submit that the specification as filed provides a legally sufficient written description for the addition of an electron transfer moiety to a nucleoside. For example, on page 20, lines 25 through page 21, line11, the specification describes the incorporation of the modified nucleosides into a growing oligonucleotide by standard synthetic techniques, referencing the Gait article in Eckstein (attached hereto as Exhibit A). In addition, to Examples 1-6, discussed above, the Examiner's attention is respectfully drawn to Figure 6 of the specification which depicts the incorporation of modified nucleoside into a growing oligonucleotide chain. The experimental conditions are provided in Example 9 (specification, at page 45, line 19 through page 46, line 10).

Accordingly, applicants submit that the written description provided in the specification is sufficient to enable one of skill in the art to synthesize the compounds of the invention. Applicants respectfully request withdrawal of the rejection of claims 12-25 under 35 U.S.C. § 112, first paragraph.

Provisional Double Patenting Rejection

Claims 12 -25 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 21-28 of co-pending Application No. 09/602,618.

Without admitting the necessity of a Terminal Disclaimer, applicants hereby submit one in an effort to expedite prosecution. A terminal disclaimer listing patent application 09/602,618 is enclosed. Applicants also enclose a copy of a letter from the California Institute of Technology stating the individual who signed the terminal disclaimer, Adam Cochran, is authorized to sign all documents relating to the filing and prosecution of trademark and patent applications on behalf of California Institute of Technology.

Applicants respectfully submit that the claims are now in condition for allowance and early notification to that effect is respectfully requested. If the Examiner feels there are further unresolved issues, the Examiner is respectfully requested to phone the undersigned at (415) 781-1989.

The Commissioner is hereby authorized to charge any additional fees, including extension fees or other relief as may be required, or credit any overpayment to Deposit Account 06-1300 (Order No. A-58762-9/RFT/RMS/RMK).

Dated: 5/8/01

Respectfully submitted,

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APPENDIX OF PENDING CLAIMS

12. A nucleoside comprising a ribose comprising a covalently attached electron transfer moiety at the 2' position.

13. A nucleoside according to claim 12 wherein said electron transfer moiety is an organic electron transfer moiety.

14. A nucleoside according to claim 12 wherein said electron transfer moiety is a transition metal complex.

15. A nucleoside according to claim 12 wherein said transition metal complex comprises ruthenium.

16. A nucleoside according to claim 12 wherein said transition metal complex comprises iron.

17. A nucleoside according to claim 12 wherein said transition metal complex comprises osmium.

18. A nucleoside according to claim 12 wherein said transition metal complex comprises rhenium.

19. A nucleoside according to claim 12 wherein said transition metal complex comprises cobalt.

20. A nucleoside according to claim 12 wherein said transition metal complex comprises palladium.

21. A nucleoside according to claim 12 wherein said transition metal complex comprises platinum.

22. A nucleoside according to claim 12 wherein said electron transfer moiety is attached via an amine group at said 2' position.

23. A nucleoside according to claim 12 wherein said electron transfer moiety is attached via a linker at said 2' position.

24. A nucleotide comprising a ribose comprising a covalently attached electron transfer moiety at the 2' position.

25. A nucleic acid comprising a nucleoside comprising a ribose comprising a covalently attached electron transfer moiety at the 2' position.

EXHIBIT A

Oligonucleotides and Analogues

A Practical Approach

Edited by

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Preface

THE chemical synthesis of oligodeoxynucleotides has come a long way since the last volume on this subject, *Oligonucleotide synthesis: a practical approach*, edited by M. Gait, was published in 1984. Still being somewhat of an art for the specialist in those days, the methodology has since undergone tremendous development. Synthesis of unmodified oligodeoxynucleotides has now become routine in most instances and certainly does not require a particular expertise any longer. This, of course, is entirely due to the automation of this process and the ongoing improvements by manufacturers in the design of synthesizers and the development of new reagents. In addition to the ease of synthesis, the realization that these compounds have a much wider application than originally anticipated makes the chemical synthesis of oligodeoxynucleotides such a fast growing area. Moreover, the discovery that certain RNAs can have catalytic activities and that RNA–protein interaction plays an important role in the control of gene expression has led to an enormous interest in the automated chemical synthesis of oligoribonucleotides as well. All these developments justify the publication of a new Practical Approach book on the chemical synthesis of oligonucleotides, including examples of their applications.

The reader will find that most of the areas included are those which have undergone considerable changes since the appearance of M. Gait's book. These include two chapters on the state of the art in automated synthesis of oligonucleotides and oligoribonucleotides, although the latter has yet to reach the level of perfection of the former. Several chapters deal with the synthesis of modified oligonucleotides. There are three chapters which describe the modification of the phosphate backbone to phosphorothioates, phosphorodithioates, and the methyl phosphonates. The importance of these lie to a considerable degree in their potential application as therapeutics. Other chapters describe the synthesis of sugar-modified oligonucleotides such as the 3'-O-methyl derivatives, the introduction of base modifications, and the attachment of reporter groups at various positions in the oligonucleotides. These latter modifications are of considerable interest to those looking for non-radioactive probes in hybridization and for suitable reporter groups for studying DNA–DNA or DNA–protein interactions.

The authors of the various chapters are all experts in their field and very often are the persons who have developed the particular area. They all submitted manuscripts of a very high standard which greatly simplified my task as editor, a job which I had accepted with some trepidation. Their enthusiastic co-operation guaranteed the delivery of manuscripts and facilitated rapid publication. I thank them all for their active support in preparing

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2

Oligoribonucleotide synthesis

MICHAEL J. GAIT, CLARE PRITCHARD, and GEORGE SLIM

1. Introduction

It is remarkable to note that, compared with a 2′-deoxyribonucleoside, the presence of a single extra hydroxyl group at the 2′-position of a ribonucleoside has given rise in recent years to so many headaches for chemists attempting to assemble oligoribonucleotide chains. For example, whereas machine-aided assembly of oligodeoxyribonucleotides has been established for some years (Chapter 1), only very recently has it been possible to assemble oligoribonucleotides satisfactorily using mechanized solid phase procedures. Part of the difficulty here has been the need to find a combination of compatible protecting groups for the ribonucleoside 2′- and 5′-hydroxyl groups. Thus in solid phase synthesis with chain assembly proceeding in a conventional 3′- to 5′-direction, 5′-terminal protecting groups must be selectively removed at every cycle of ribonucleotide addition, whereas 2′-protecting groups must remain intact throughout all steps of oligoribonucleotide assembly and must be removed specifically at the end of the synthesis without leading to chain migration or internucleotidic cleavage. Further problems stem from the less facile coupling reactions and hence slightly poorer coupling efficiences obtained hitherto in oligoribonucleotide synthesis. In addition, oligoribonucleotides are highly susceptible to degradation by ribonucleases, which are ubiquitous and difficult to remove, and therefore the utmost care must be taken during all purification steps.

Completely satisfactory solutions to all these problems are not yet available. Nevertheless, it has now become possible to synthesize oligoribonucleotides of moderate length in reasonable yield and purity by machine-aided methods and by use of commercially available reagents. We now present protocols that have worked reasonably well in our hands but we are mindful that further improvements are likely to follow soon in terms of better reagents and methods. We therefore concentrate where possible on basic techniques which should still be of relevance as new materials become available.

1.2 Basic chemistry of oligoribonucleotide synthesis

The solid phase strategy for synthesis of oligoribonucleotides is very similar to that employed in the preparation of oligodeoxyribonucleotides (see Chapter 1). Thus a suitably protected nucleoside derivative is attached to a controlled pore glass support via a succinate linker. Cycles of addition of nucleotide units are then carried out by removal of 5′-terminal protecting groups, coupling to the next nucleotide unit, capping and oxidation. The completed oligonucleotide is then cleaved from the support, and phosphate and heterocyclic base protecting groups are removed by treatment with ammonia solution. The final step is the complete removal of 2′-protecting groups, followed by purification of the oligonucleotide by gel electrophoresis or by HPLC.

Many alternative 2′-protecting groups have been explored for use in oligoribonucleotide synthesis. The most reliable groups in terms of their selectivity of introduction and cleanness of removal without formation of side products are acid-labile acetal groups, such as tetrahydropyran-1-yl (1) and 4-methoxytetrahydropyran-4-yl (2). Unfortunately their use is not totally compatible with conventional 5′-dimethoxytrityl groups because of partial loss during 5′-deprotection (3). One solution to this problem is the use of the modified acetal 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) as a 2′-protecting group (4). The Fpmp group is reported to be stable during the non-aqueous acidic treatment required to remove 5′-dimethoxytrityl groups, but is readily removed under aqueous conditions at pH 2. Hopefully, recently reported successes in oligoribonucleotide synthesis using this protecting group (5) will be translated into a commercially viable route (Cruachem, UK).

An alternative solution to the problem of 2′- and 5′-protecting group compatibility is the replacement of dimethoxytrityl as a 5′-protecting group with a group that is removed under non-acidic conditions. In this context, both the levulinyl group (6) and the 9-fluorenylmethoxycarbonyl group (7) have been successfully utilized in conjunction with an acid-labile 2′-protecting group for synthesis of oligoribonucleotides up to 25 residues in length. Neither of these routes has thus far attracted much commercial interest principally because such ribonucleotide materials cannot be used interchangeably with conventional 5′-dimethoxytrityl-containing deoxyribonucleotide materials to make mixed RNA–DNA sequences.

The method which has gained most favour amongst commercial suppliers is that which has been developed principally by Ogilvie and co-workers over many years and which utilizes the t-butyldimethylsilyl group for 2′-protection (8). Only more recently, however, has the use of this protecting group become reasonably accepted and *Figure 1* shows the currently preferred route for synthesis of the ribonucleoside 3′-phosphoramidite derivatives. First, it

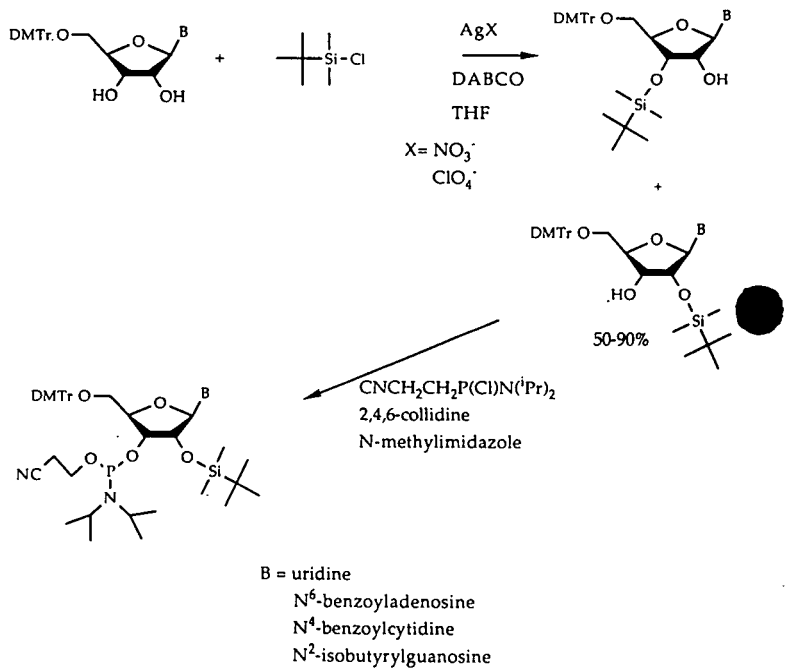

Figure 1. Steps in synthesis of the four ribo amidites.

was necessary for a selective procedure of 2′-silylation to be developed for each of the four 5′- and N-protected ribonucleosides (9). Secondly, the danger of migration of the 2′-silyl group to the 3′-position under mildly alkaline conditions (10) needed to be recognized and appropriate precautions taken. This is particularly important in the case of 3′-phosphitylation where under the standard conditions previously recommended (N,N-diisopropylamino, 2-cyanoethyl chlorophosphite in the presence of diisopropylethylamine and N,N-dimethylaminopyridine) (11) some migration may be expected. Very recently it has been shown that replacement of base and catalyst by 2,4,6-collidine and N-methylimidazole respectively leads to isomeric purity levels of the ribonucleoside phosphoramidites of > 99.95% (12). It should also be noted that the currently used heterocyclic protecting groups (benzoyl for A, benzoyl for C, and isobutyryl for G), developed many years ago by Khorana and co-workers to meet the needs of solution phase oligonucleotide synthesis, require the use of quite harsh ammoniacal conditions for their removal at the end of oligonucleotide assembly. Under these conditions,

there is a danger of partial loss of *tert*-butyldimethylsilyl groups, which can lead eventually to reductions in synthetic yield due to chain cleavage reactions. Although adjustments to the conditions of ammonia treatment have alleviated this problem somewhat, the use of protecting groups removable under milder conditions may be preferable in future.

Another requirement for oligoribonucleotide synthesis by modern solid phase chemistry is a solid support to which is attached one of the four ribonucleosides to act as a 3′-end of an oligoribonucleotide chain (*Figure 2*). The linkage to the support is via a succinate group. This is introduced on to a ribonucleoside derivative by treatment with succinic anhydride largely as previously outlined for deoxyribonucleosides (11, 13). The nucleoside succinate derivative is then coupled to a controlled pore glass support via intermediate preparation of the corresponding pentachlorophenyl (11) or pentafluorophenyl (C. Pritchard, unpublished) ester. It should be noted that because the nucleoside attached to the support becomes the 3′-residue of an oligoribonucleotide, it does not matter if the succinate linkage is formed through the 2′- or 3′-position, since both positions become deprotected in the final oligonucleotide. Usman *et al.* (11) suggested that the unwanted 3′-*tert*-butyldimethylsilyl nucleoside derivatives prepared as by-products during the preparation of ribonucleotide monomers (*Figure 1*) could be effectively used for succinate derivation. Alternatively, succinylation of 2′(3′)-acetyl ribonucleosides is quite acceptable. In either case, any migration of protecting groups at this 3′-terminus is immaterial.

Assembly of oligoribonucleotide chains takes place in a very similar way to that already described for oligodeoxyribonucleotides (see Chapter 1). First, terminal 5′-dimethoxytrityl groups are removed by treatment with trichloroacetic acid solution in dichloromethane (*Figure 3*). Then, coupling of a ribonucleoside phosphoramidite derivative is carried out in acetonitrile

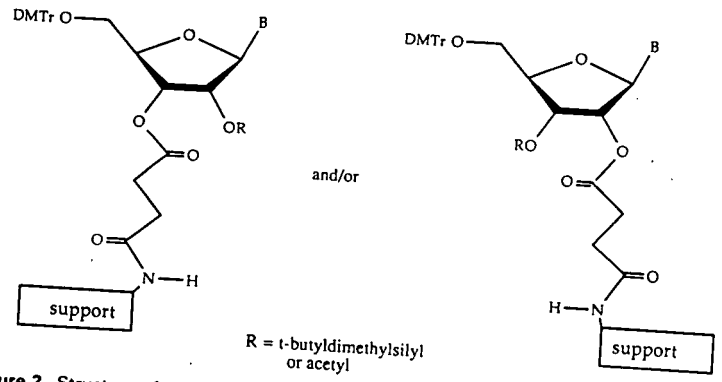

Figure 2. Structure of solid supports functionalized with ribonucleosides.

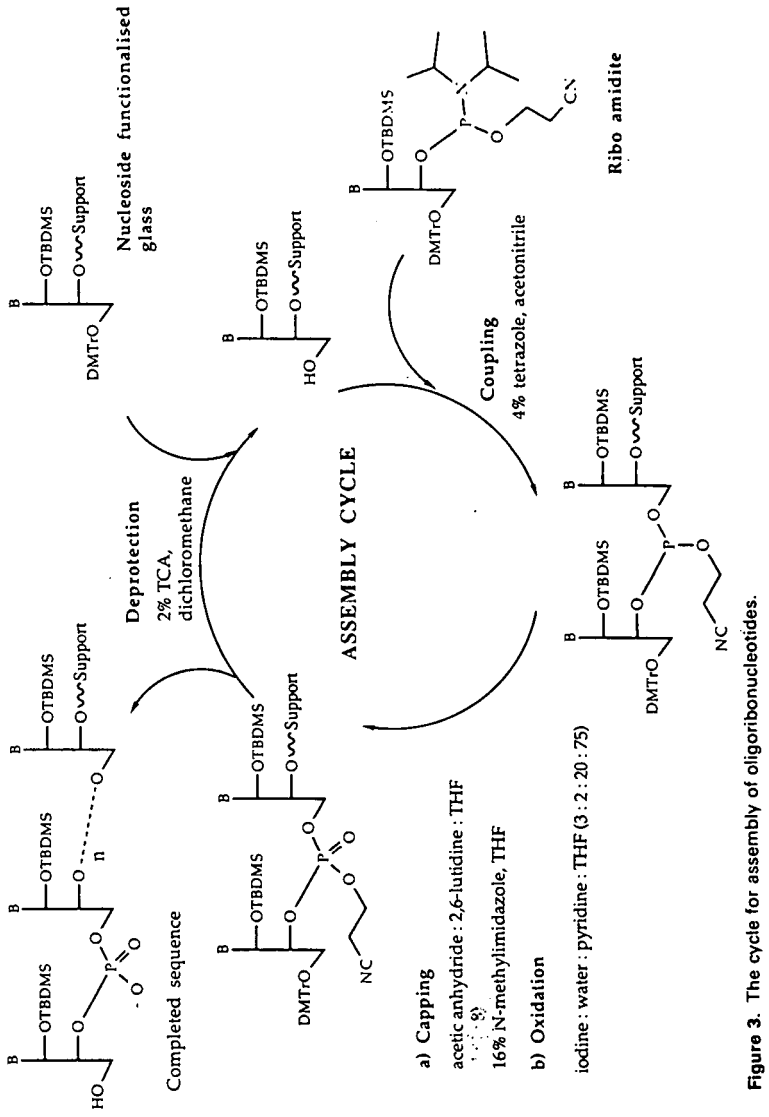

Figure 3. The cycle for assembly of oligoribonucleotides.

solution using tetrazole as catalyst. It should be noted that such coupling reactions are much slower than those between deoxyribonucleotides. The reason for this is not fully understood. It cannot be ascribed totally to the proximity of a bulky 2′-protecting group, since the much less hindered 2′-O-methyl derivatives give rise to similarly slow couplings (Chapter 3). The more activated 5-(4-nitrophenyl)tetrazole has been used as a catalyst to increase the speed of ribonucleotide coupling reactions (5, 7). However, it is our experience that the sparing solubility of this reagent in acetonitrile solution (*c.* 0.1 M) causes some problems in routine use on commercial DNA synthesizers due to lack of consistency in coupling reactions, perhaps because of partial precipitation of the reagent when forced through narrow tubing. At the time of writing, therefore, tetrazole is the preferred catalyst despite the slower internucleotide couplings.

The cycle of nucleotide addition is completed by a 'capping' reaction, involving treatment of the support with acetic anhydride and N-methylimidazole in the presence of 2,6-lutidine in tetrahydrofuran followed by oxidation of intermediate phosphite triesters to phosphate triesters using iodine in aqueous pyridine solution.

Further cycles of assembly involving terminal deprotection, coupling, capping, and oxidation are carried out to elaborate the desired sequence. All these cycles are usually carried out using a commercial DNA/RNA synthesis machine. Finally, 5′-terminal dimethoxytrityl groups are removed using a further trichloracetic acid treatment, since it is our experience that the conditions used later for 2′-deprotection of the oligoribonucleotide, treatment with tetrabutylammonium fluoride (TBAF), give rise to partial loss of 5′-dimethoxytrityl groups and thus it is better to remove these groups completely immediately after oligonucleotide assembly.

Removal of phosphate protecting groups (2-cyanoethyl), heterocyclic base protecting groups (benzoyls and isobutyryl), and cleavage of the oligonucleotide from the glass support is achieved by heating in ammonia solution (*Figure 4*). Here the use of totally aqueous ammonia (see Chapter 1) is not recommended since there is substantial loss of 2′-*tert*-butyldimethylsilyl groups. This leads to some cleavage of the internucleotide linkages by attack of the liberated 2′-hydroxyl groups on neighbouring phosphotriesters or phosphodiesters (14). Use of concentrated ammonia/ethanol (3:1) is far better in this respect (10,13) leading to only a small amount of oligonucleotide degradation. However, very recently the use of anhydrous ammonia in ethanol has been reported to result in no degradation at all (12). Unfortunately this reagent is both hygroscopic and rather volatile and must be prepared freshly, which makes it less attractive for automated use.

Finally, 2′-*tert*-butyldimethylsilyl protecting groups are removed using 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran. This process requires a full 24 h for completion but is highly specific (14). However, great care must be taken to ensure that the TBAF reagent is completely removed

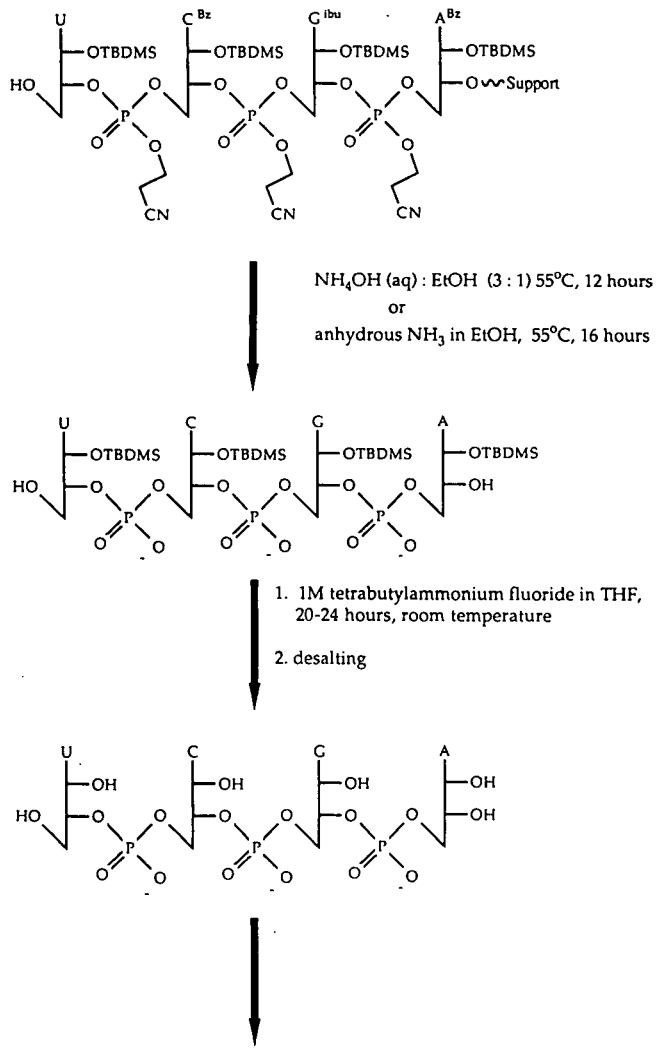

Figure 4. Steps in deprotection and purification of oligoribonucleotides.

subsequently in order to preserve the oligonucleotide chain intact (see below). The deprotected oligonucleotide is now ready for purification and analysis.

3. Materials and reagents

Practically all reagents and materials for oligoribonucleotide synthesis are commercially available but at this early stage of development of oligoribonucleotide synthesis chemistry, not all suppliers' materials are of sufficient quality. We recommend the following:

Ribonucleoside phosphoramidites Milligen/Biosearch

5′-O-(4,4′-Dimethoxytrityl)-2′-O-*tert*-butyldimethylsilyl-6-N-benzoyl-adenosine-3′-O-(2-cyanoethyl)-N,N′-diisopropylphosphoramidite
0.5 g Ribo Amidite A GEN 067001

5′-O-(4,4′-Dimethoxytrityl)-2′-O-*tert*-butyldimethylsilyl-4-N-benzoyl-cytidine-3′-O-(2-cyanoethyl)-N,N′-diisopropylphosphoramidite
0.5 g Ribo Amidite C GEN 067011

5′-O-(4,4′-Dimethoxytrityl)-2′-O-*tert*-butyldimethylsilyl-2-N-isobutyryl guanosine-3′-O-(2-cyanoethyl)-N,N′-diisopropylphosphoramidite
0.5 g Ribo Amidite G GEN 067021

5′-O-(4,4′-Dimethoxytrityl)-2′-O-*tert*-butyldimethylsilyluridine-3′-O-(2-cyanoethyl)-N,N′-diisopropylphosphoramidite
0.5 g Ribo Amidite U GEN 067031

Nucleoside-functionalized controlled pore glass supports

(For self-packing of columns)	Peninsula Labs
DMT-r-A(bz)-2′-tBuSiR-CPG	NR2023S
DMT-rC(bz)-2′-tBuSi-CPG	NR2024S
DMT-rG(ibu)-2′-tBuSi-CPG	NR2027S
DMT-rU-2′-tBuSi-CPG	NR2026S

(Prepacked columns for use on Milligen/Biosearch machines are also available from Milligen)

Other reagents for oligoribonucleotide assembly

These are identical to those normally used for DNA synthesis:
Activator: 4% tetrazole/acetonitrile
Terminal deprotection: 2% trichloracetic acid/dichloromethane
Capping: acetic anhydride/2,6-lutidine/THF (1:1:8)
16% 1-methylimidazole/THF
Oxidation: iodine/water/pyridine/THF (3:2:20:75)
Anhydrous acetonitrile (for dissolution of amidites)

We recommend that these reagents are purchased according to the specifi-

cations of the supplier of DNA/RNA synthesizer equipment. In our case we have used an Applied Biosystems 380B Synthesizer and Applied Biosystems reagents throughout. HPLC grade acetonitrile (e.g. from Rathburn, Romil, Fisons etc.) can be used for all intermediate washing steps.

Deprotection reagents

(i) *Ammonium hydroxide/ethanol (3:1)*

35% ammonia solution (Analar) BDH, Poole UK
Absolute ethanol (Analar) BDH, Poole UK

Mix the reagents just prior to use in the synthesizer and transfer to an appropriate bottle. We recommend that the reagent is made up freshly each day, since some loss of ammonia is observed upon standing at room temperature. We have very recently attempted to use anhydrous ammonia in ethanol for deprotection (12). This is prepared by bubbling ammonia gas through absolute ethanol which is cooled on ice. Care must be taken to exclude moisture in this procedure. Also the reagent is very unstable and should be stored at −20 °C for a maximum of 3–4 days. We also recommend that the reagent is not connected to the synthesis machine but used manually by syringe injection into the column after disconnection from the machine. Very preliminary results suggest that yields are at least as good as in the case of partially aqueous ethanolic ammonia and possibly better.

(ii) *2′-Hydroxyl deprotection*

1 M tetrabutylammonium fluoride in THF (less than 5% water) Aldrich Chemical Co.

Volatile buffers

(i) *2 M triethylammonium bicarbonate (TEAB) solution*

Add triethylamine (28 ml, BDH Analar) to sterile water (50 ml) and bubble carbon dioxide gas through the mixture with occasional swirling until the triethylamine is completely dissolved. Continue bubbling until the pH of the solution drops below 8. Make up to 100 ml with sterile water and store in a sterile bottle at 4 °C. Dilute as necessary with sterile water to the required molarity.

(ii) *0.1 M triethylammonium acetate solution*

Add to sterile water (500 ml) triethylamine (6.7 ml) and glacial acetic acid (2.8 ml, analytical grade, Fisons). After thorough mixing, adjust the pH to 7.0 by addition of either a few drops of acetic acid or a few drops of triethylamine as required.

Reagents used in oligonucleotide purification

(i) *Sterile water*

A major source or ribonucleases derives from the use of insufficiently pure water. We recommend that double distilled and autoclaved water is used for

all buffers and aqueous reagent that come into contact with oligoribonucleotides (see also precautions against ribonuclease cleavage, Section 5).

(ii) *HPLC*:

- Reagents

formamide (Analar)	BDH
water (HiPerSolv)	BDH
potassium dihydrogenorthophosphate (Analar)	BDH
potassium hydroxide (Analar)	BDH
acetonitrile (HPLC grade S)	Rathburn
ammonium acetate (Analar)	BDH

- Preparation of ion exchange buffers
  Make a stock solution of 1.0 M potassium dihydrogenorthophosphate (KH_2PO_4) with pH adjusted to 6.3 by careful addition of potassium hydroxide. Solvent A (15 mM phosphate): 15 ml KH_2PO_4 stock solution + 600 ml water + 900 ml formamide; Solvent B (300 mM phosphate): 300 ml KH_2PO_4 stock solution + 100 ml water + 600 ml formamide.
  Notes: (1) Higher phosphate concentrations cannot be used with this system because of the danger of precipitation of potassium phosphate; (2) it is best to use the same batch of formamide in both the buffers to ensure a good UV baseline in gradient elution; and (3) 40% acetonitrile may be used instead of formamide as disaggregant but care should be taken here to avoid precipitation of potassium phosphate in pump B by ensuring that it is always pumping, even if at low speed.

- Preparation of reversed phase buffers
  Solvent A: 0.1 M ammonium acetate solution (pH unadjusted).
  Solvent B: 0.1 M ammonium acetate solution/acetonitrile (6:4).

- Columns

Partisphere 5-SAX (0.46 × 12.5 cm) analytical cartridge	Whatman
Partisil 10-SAX (1.0 × 25 cm) semi-preparative column	Hichrom, Reading, UK or Whatman
µ-Bondapak C18 (0.39 × 30 cm) analytical column or (0.78 × 30 cm) semi-preparative column	Waters/Millipore

(iii) *Desalting*

Sephadex G25 or Sephadex NAP columns	Pharmacia
Qiagen pack 500 strong anion exchange cartridges	Hybaid Ltd, Middlesex, UK
Dialysis tubing (Visking)	Medicell International

4. Assembly of oligoribonucleotide chains and deprotection

4.1 Machine and reagent preparation

We have carried out all assemblies on an Applied Biosystems 380B DNA Synthesizer, although other manufacturers machines are also applicable in general. Solutions of ribo amidites are made up at 0.1 M concentration by direct injection of anhydrous acetonitrile into 0.5 g bottles as follows: A 5.2 ml, G 5.3 ml, C 5.4 ml, U 6.0 ml. The bottles are now connected to the machine (*Note that if the machine has previously been used for deoxynucleotide synthesis, very careful washing of the amidite delivery lines is necessary. A single bottle change procedure may not be sufficient in all cases.*)

4.2 Column packing

Currently, prepacked columns are not available for oligoribonucleotide synthesis using Applied Biosystems machines. This is not a difficulty since empty columns can be packed very simply (*Protocol 1*).

Protocol 1. Packing of columns

1. Weigh out the appropriate amount of support on a micro-balance. For 1 µmol scale this will be about 30 mg of 33–35 µmol/g nucleoside functionalized support. Do not fill an ABI standard column with more than 40 mg of support, since there is insufficient mixing during flow and synthesis results become poor.
2. Using a small funnel, transfer the support into an empty column and crimp or snap close.
3. Test the column for leaks by connecting to the synthesizer and flow in both directions using acetonitrile.

4.3 Assembly cycle

For oligoribonucleotide assembly, we use an identical cycle to that used for DNA synthesis on 1 µmole scale *except* that the wait step of the coupling reaction is increased to 600 s (as opposed to 30 s for DNA synthesis).

To check the efficiency of coupling, use exactly the same procedure as is recommended for assembly of DNA chains (ABI under bulletin 13).

- Dilute each TCA eluate (collected by fraction collector in glass tubes) to 50 or 100 ml with 0.1 M toluene-*p*-sulphonic acid in acetonitrile.
- Measure the absorbance at 498 nm (ε 71 700) and compare for each synthesis cycle as a percentage of the previous cycle result.

In our experience, the efficiences are about 98% for U or G couplings, 96% for A, and 95% for C couplings. Hopefully these values will improve as commercial materials become of higher quality.

4.4 Deprotection

The first part of the deprotection is pre-programmed on the 380B Synthesizer, (end procedure) but this step can also be carried out manually by connecting the column to a syringe having a Luer fitting (*Protocol 2*).

Protocol 2. Deprotection of assembled oligoribonucleotide chains

1. Treat the support with the ammonia/ethanol deprotection reagent in 5 batches for 1450 s each. Note that this is double the time normally allowed for aqueous ammonia treatment in oligodeoxyribonucleotide synthesis. Collect the five ammoniacal eluates together in a small screw-capped glass vial.
2. Seal the vial and heat at 55 °C (water bath, oven, or heating block) for 8–12 hours.
3. Freeze the sample in dry ice/acetone and then lyophilize. We recommend a Savant SpeedVac Concentrator for this purpose, since the vial can be placed directly in one of the rotor buckets. The freezing to low temperature prevents 'bumping' of the ammonia solution.
4. Resuspend the residue in 1 M TBAF solution (1 ml).
5. Seal the vial again and keep in the dark at room temperature for 20–24 h. Swirl occasionally to ensure that a homogeneous solution is obtained (NB. If total solubility is not achieved eventually, the silyl removal may not proceed to completion. Repeat treatment perhaps at lower TBAF concentration may be needed.)
6. To quench the reaction, add to the mixture 0.1 M triethylammonium acetate solution (5 ml, if dialysis is used for desalting or 1 ml if gel filtration is used, see below) or 0.1 M TEAB solution (10 ml) (if Qiagen cartridge desalting is used) and the sample is now ready for desalting.

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5. Desalting and purification of oligoribonucleotides

5.1 Precautions to avoid ribonuclease contamination

The importance of good handling techniques and care in preparation of buffers, reagents, and apparatus cannot be overstressed. Ribonucleases are ubiquitous and the slightest trace can give rise to degradation of the oligoribonucleotide. All water used should be sterile (see Section 3) and reagents should be of the highest purity. Wherever possible, sterile disposable

plastic tips and tubes should be used for storage and handling of solutions of oligoribonucleotides. If glassware must be used, wash well with chromic acid, rinse exhaustively in glass-distilled water, and bake in the hottest possible oven (or autoclave). Disposable plastic gloves should be used at all times when handling RNA. Separate HPLC columns should be used for RNA purification and for enzymatic analysis of RNA (Section 6).

5.2 Desalting

It is vital to remove all traces of TBAF before attempting evaporation of the deprotected oligoribonucleotide sample. In our experience, there is great danger of degradation of the oligoribonucleotide chain during evaporation and therefore we recommend an initial desalting step. Three methods have been successfully used in our laboratory.

5.2.1 Dialysis against water (*Protocol 4*)

The dialysis tubing must first be very carefully prepared (15) (*Protocol 3*). Gloves should be worn.

Protocol 3. Preparation of dialysis tubing

1. Boil appropriate length pieces of tubing in a large volume of 2% sodium bicarbonate and 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA), for 10 min.
2. Rinse the tubing thoroughly with sterile water.
3. Boil the tubing for 10 min in sterile water. This step can be replaced by 'wet' autoclaving in a loosely capped jar of sterile water, which is recommended if possible.
4. Cool and store at 4 °C under sterile water.
5. Before use, wash inside and outside of the tubing with sterile water.

Protocol 4. Desalting by dialysis

1. Transfer the oligonucleotide solution into a section of dialysis tubing and seal the ends.
2. Place the dialysis tube in a flask containing 5 litres of gently stirred distilled water (it is not essential to use sterile water on the outside of the dialysis bag).
3. Allow to equilibrate for at least 2 h.
4. Change the external water three times. It is recommended that the minimum time be used for dialysis (usually 8–16 h) to avoid the possibility of RNA degradation.

5.2.2 Strong anion-exchange cartridge method (12) (*Protocol 5*)

An alternative to ion exchange cartridge desalting is the use of a reversed phase cartridge. We have currently insufficient experience in their routine use for desalting oligoribonucleotides to make a positive recommendation, but we have found both OPC cartridges (Applied Biosystems) and Poly-Pak cartridges (Glen Research) are useful for oligodeoxyribonucleotide desalting.

Protocol 5. Desalting by use of ion exchange cartridge

1. Using a disposable syringe or via gravity flow elution, apply the oligonucleotide sample, which must be less than 0.1 M in TBAF, to a Qiagen pack 500 cartridge which has been pre-washed with 15 ml 0.1 M TEAB containing 0.15% Triton X-100.
2. Collect the eluate and reapply this to the Qiagen cartridge to ensure complete absorption of the oligonucleotide.
3. Wash the column with 0.1 M TEAB solution (7 ml) and then elute the oligonucleotide from the cartridge using 2 M TEAB (12 ml).
4. Collect the eluate and lyophilize.

5.2.3 Sephadex gel filtration (11) (*Protocol 6*)

Prepacked sterile Sephadex NAP-10 columns can also be used for this desalting step (*Protocol 7*).

Protocol 6. Desalting by conventional gel filtration

1. Load the sample of oligonucleotide on a Sephadex G25F column (30 × 1 cm) made up in sterile water (Note that the glass column should be acid washed and the Sephadex should be autoclaved).
2. Elute the column with water by gravity flow or by a peristaltic pump.
3. Collect fractions in sterile microfuge tubes.
4. Measure the UV absorbance at 260 nm. The first eluting peak at the void volume is the oligonucleotide.
5. Evaporate fractions using a SpeedVac Concentrator.

Protocol 7. Desalting using Sephadex NAP-10 columns

1. Freeze the oligoribonucleotide sample and lyophilize just long enough to reduce the volume to less than 1 ml.
2. Dilute with sterile water to exactly 1 ml.

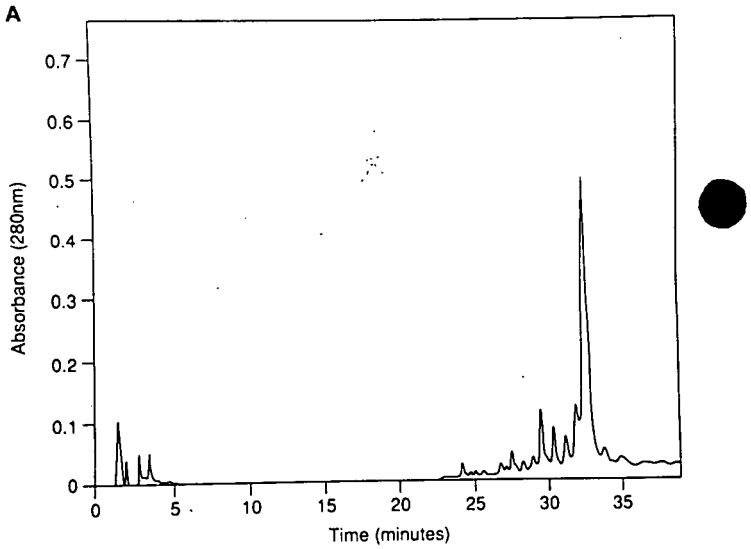
Protocol 7. *Continued*

3. Apply to a prewashed (15 ml of sterile water) NAP-10 column.
4. Elute with sterile water (1.5 ml) by gravity flow and collect the eluate.
5. Evaporate using a SpeedVac Concentrator.

5.3 HPLC purification of oligoribonucleotides

5.3.1 Ion exchange HPLC

The advantage of ion exchange systems in purification of oligonucleotides is that resolution is by formal negative charge which increases as the length of the oligonucleotide increases. Thus, the longer the oligonucleotide the later it is eluted. This makes identification of the desired product oligonucleotide in solid phase synthesis quite easy. We have found that the best analytical ion exchange columns to date for both oligodeoxyribo- and oligoribo-nucleotides are Partisphere 5-SAX (7, 16) (*Protocol 8*) and for preparative isolation, Partisil 10-SAX (7, 17) (*Protocol 9*). A typical separation on an analytical column is shown in *Figure 5A* for the 13mer r(GCCUGU)d(C)r(AGUCCC) (see Section 7.1 for mixed ribo–deoxyribooligonucleotide synthesis). A preparative separation of the same 13mer is shown in *Figure 5B* and an analytical check of purity after storage of the 13mer for 3 months at −20 °C as a solid is shown in *Figure 5C*.



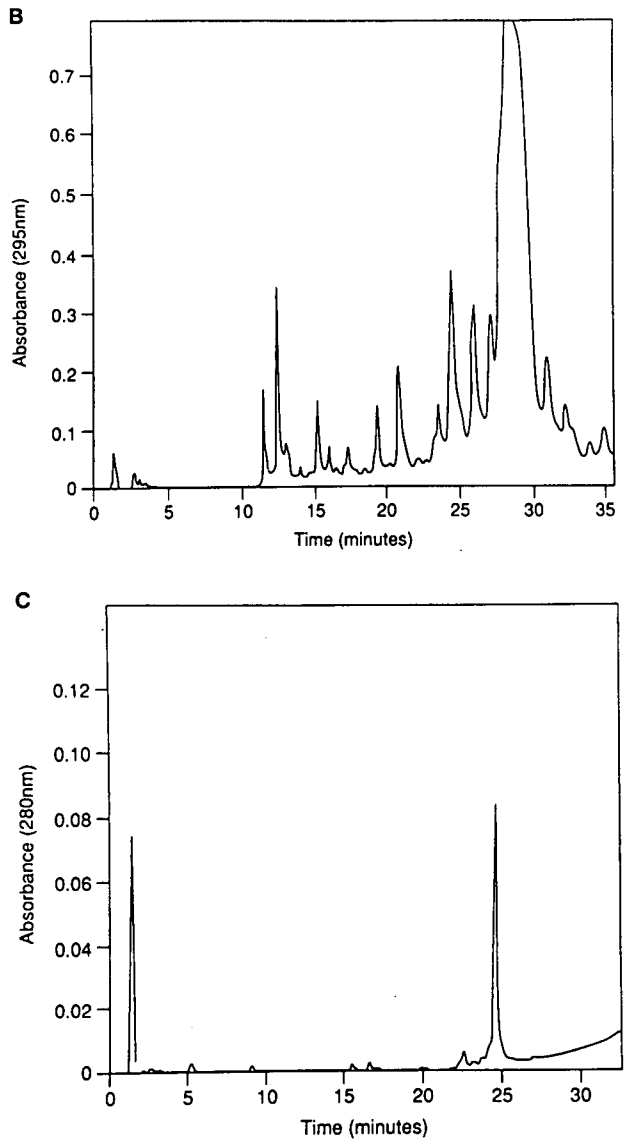


Figure 5. Ion exchange separations of the 13mer r(GCCUGU)d(C)r(AGUCCC). **(A)** Analytical separation on Partisphere 5-SAX. Conditions as in text. **(B)** Preparative separation on Partisil 10-SAX. Conditions as in text. **(C)** Analytical check on purity after storage using Partisphere 5-SAX. Conditions as in text except salt gradient flattened.

Since oligoribonucleotides are retained on ion exchange columns considerably longer than oligodeoxyribonucleotides, we have found the practical limit of resolution to be up to about 27 residues. This is in contrast to 2′-methoxytetrahydropyranyl-protected oligoribonucleotides which are retained to about the same extent as oligodeoxyribonucleotides (7).

Before HPLC the sample should be prepared as follows:

- Dissolve the oligoribonucleotide sample from a 1 μmol scale synthesis in 1 ml of sterile water just prior to purification.
- Clarify the sample by microfuge centrifugation using an Ultrafree-MC 0.22 μm filter unit (Millipore, UFC3 OGV 00), which takes 400 μl aliquots per centrifugation, or by passage through a 0.2 μm Acrodisc syringe filter (Gelman Sciences). In the latter case an extra 0.5–1 ml water must be passed through to flush the filter unit.

Protocol 8. Analytical separations of oligoribonucleotides on Partisphere 5-SAX

1. Set the flow rate to 1.0 ml/min.
2. Set the UV monitor to 280 nm on the 1.0 scale. Wavelengths lower than 270 nm cannot be used because of the UV absorption of formamide.
3. Use a starting gradient of 0–100% B over 30 min initially to gauge the success of assembly, but the gradient subsequently can be flattened if desired.
4. Inject 10 μl of sample and run the gradient. The last peak to emerge should be the desired oligonucleotide if the synthesis has proceeded correctly.

Protocol 9. Preparative separations of oligoribonucleotides on Partisil 10-SAX

1. Set the flow rate: to 2.5 ml/min.
2. Set the UV monitor to 280 nm on the 2.0 scale.
3. Use a starting gradient of 0–100% B over 30 min for the first injection.
4. Inject about 30 μl of sample and run the gradient.
5. Observe the elution position of the major (last peak) and decide if the gradient should be flattened to achieve optimal separation.
6. Adjust the UV monitor sensitivity to the 1.0 or 2.0 scale at 295 nm and inject about 200–250 μl of sample.
7. Collect the product in the desired peak as it is eluted. 4–5 injections

Protocol 9. *Continued*

should be sufficient for purification of all the material from a 1 μmol scale synthesis.

8. Remove the salt and formamide by dialysis against water (see Section 5.2) and isolate the product by lyophilization.

5.3.3 Reversed phase HPLC

This method of separation is not recommended for fully deprotected oligonucleotides as a first step procedure because the elution position of the desired oligonucleotide cannot be predicted with accuracy. However it may be useful as a second purification step after an initial separation using strong anion-exchange and especially if ultra-high purity oligoribonucleotides are required (*Protocol 10*). *Warning*: it is our experience that some oligoribonucleotides with stable secondary structures do not give single sharp peaks on reversed phase HPLC.

Protocol 10. Reversed phase separations of oligoribonucleotides

1. Set the flow rate to 1.5 ml/min.
2. Set the UV monitor to 260 nm.
3. Try initially a gradient from 0–100% B over 30 min to establish the approximate elution position.
4. Inject a sample of oligoribonucleotide (after purification by ion exchange HPLC or by PAGE) and run the gradient.
5. Observe the elution profile and decide if the product is eluting mostly as a single peak. If desired, the gradient may then be flattened considerably for optimal resolution.
6. Preparative separations should only be attempted if analytical HPLC shows predominantly a single component with no sign of secondary structural interference. In this case use a flow rate of 3 ml/min and a semi-preparative column.

5.4 Polyacrylamide gel electrophoresis (PAGE)

Methods for purification of oligoribonucleotides by PAGE (*Protocol 11*) are very similar to those used for oligodeoxyribonucleotides.

Protocol 11. Preparative separations of oligoribonucleotides by PAGE

1. Pour a 200 × 400 mm gel between glass plates having 1.5 mm spacers. We recommend 20 mm slots as being most convenient for the comb. For oligoribonucleotides up to 20 residues, a 20% gel should be used, whereas for 20–40 residues a 15% gel is preferable (7 M urea, 1 × TBE, acrylamide/bis-acrylamide (100: 2.5)).
2. Dilute the desalted oligoribonucleotides obtained from a 1 μm scale synthesis (approximately 40–50 A_{260} units in 1 ml sterile water) with 1 ml dye mix (8 ml formamide, 100 μl 0.5 M EDTA, 2 mg bromophenol blue made up to 10 ml with sterile water).
3. Load on to the gel (at least 10 × 20 mm slots).
4. Electrophorese in 1 × TBE buffer at 30–40 W for 2–3 h until the bromophenol blue dye reaches close to the bottom of the gel.
5. Remove gel from both glass plates by transferring on to a single sheet of Saranwrap.
6. Locate the bands by UV shadowing. This involves shining UV light at 254 nm on to the gel placed on an autoradiography screen (e.g. Dupont Cronex Lightning Plus-ZK screen) using a special dark box to avoid the health hazard posed by short-wave UV light. Permanent records can be obtained by Polaroid photography. *Figure 6* shows the UV shadowing of a 29mer oligoribonucleotide.
7. Excise bands and transfer to microfuge tubes.
8. Soak the gel pieces (without crushing) in sterile 0.5 M ammonium acetate, 1 mm EDTA, 0.5% sodium dodecyl sulphate (SDS) at room temperature for 6–18 h. Be sure all the gel pieces are covered by the elution buffer.
9. Carefully suck off the liquid and repeate the soaking.
10. Save and combine the gel extract solutions and centrifuge or filter to remove any small gel pieces.

The oligoribonucleotide can then be recovered by a butanol extraction procedure (20) (*Protocol 12*). This procedure removes all buffers, urea, and SDS. If desired, the oligoribonucleotide can then be precipitated with ethanol or subjected to reversed phase cartridge purification.

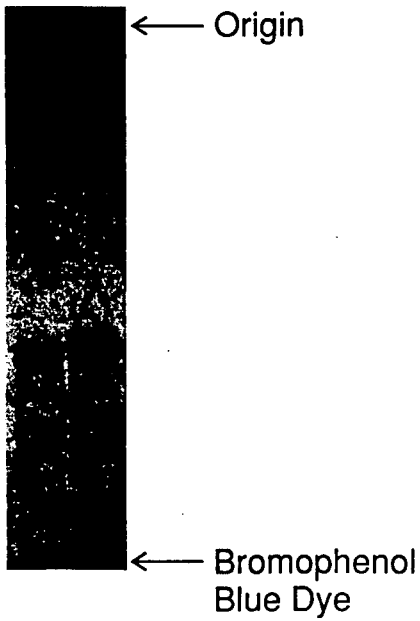


Figure 6. Polaroid photograph of UV (254 nm) shadowed preparative 15% polyacrylamide gel showing two adjacent 20 mm slots of separation of a crude 29mer oligoribonucleotide synthesis.

Protocol 12. Butanol extraction procedure for oligoribonucleotide recovery

1. Fill the microfuge tube with n-butanol.
2. Vigorously shake for 30 s and centrifuge for 1 min.
3. Remove the butanol (top) phase.
4. Repeat the extraction several times, each time filling the tube with n-butanol. Finally a pellet will be formed.
5. Redissolve the pellet in water (200 µl) and dry again to a pellet by a single n-butanol extraction.
6. Repeat step 5 three times.

6. Enzymatic analysis of oligoribonucleotides

This should no longer prove necessary on a routine basis since the quality of materials now available commercially is such that under normal circumstances wrong linkages (2′–5′) or modified bases should not be present in the final oligoribonucleotide. However, if there is any doubt in any particular circumstance, we recommend total enzymatic digestion with ribonucleases followed by phosphatase treatment, and then separation of the resultant nucleosides by reversed phase HLPC. The choice of ribonucleases is somewhat dependent on the sequence of the oligoribonucleotide. We prefer to use a mixture of RNase A, RNase T1 and RNase T2 which appears to be sufficient for most cases (*Protocol 13*). It is a good idea to check the quality of the ribonuclease preparations by carrying out a control digestion of a 2′–5′ linked di- or trinucleotide (e.g. rA (2′–5′)A which is available from Sigma). Such compounds should be totally resistant to digestion.

Protocol 13. Enzymatic digestion and analysis of oligoribonucleotides

1. Treat 0.2 A_{260} units of oligoribonucleotide dissolved in 0.05 M ammonium acetate, 0.002 M EDTA (pH 4.5; 40 µl) with RNase A (Sigma, 0.25 mg/ml, 5 µl), RNase T1 (Sigma, 50 units/ml, 5 µl), and RNase T2 (50 units/ml; 5 µl) at 37 °C for 16 h).
2. Evaporate to dryness (SpeedVac).
3. Dissolve the residue in 0.1 M Tris–HCl, 0.01 M $MgCl_2$ (pH 8.5; 50 µl).
4. Add calf intestinal alkaline phosphatase (Boehringer, 28 units/ml, 1 µl) and leave at 37 °C for 16 h.
5. Carry out reversed phase HPLC of the product essentially as described in section 5.3 except use isocratic elution (5% buffer B) to separate the four ribonucleosides followed by gradient elution to check for the absence of longer oligonucleotides. A typical analysis of total digestion of a 27mer oligoribonucleotide is shown in *Figure 7*.

7. Special applications

7.1 Mixed RNA–DNA oligonucleotides

These can be made very simply without significant changes to the protocols or reagents. In chain assembly steps whenever a deoxyribonucleotide amidite is required to be added, the normal deoxy cycle of addition (shorter coupling time) is programmed (Chapter 1). However, the synthesizer must contain sufficient ports for all the different deoxy and ribo amidites required for the

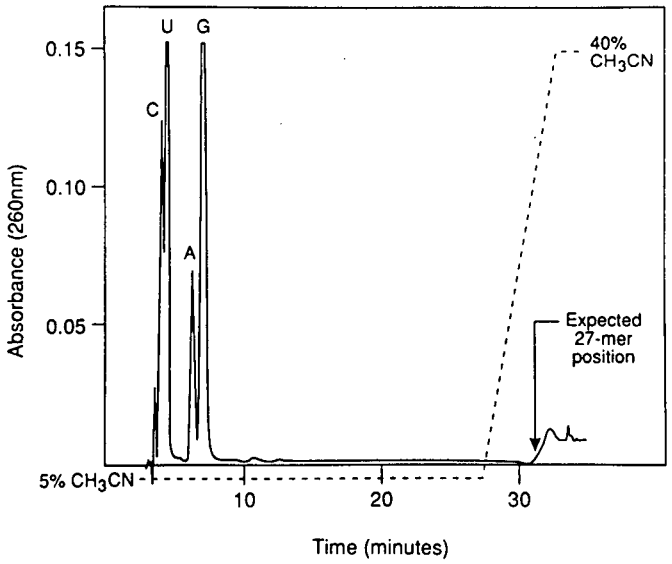


Figure 7. Reversed phase HPLC of the enzymatic digestion products of a 27mer oligoribonucleotide. Conditions of digestion and chromatography are as described in the text.

assembly. Similarly if a deoxyribonucleotide is to be introduced at the 3′-end, use a prepacked deoxynucleoside column (Chapter 1). For deprotection and work-up, follow the procedures outlined for oligoribonucleotides.

7.2 Phosphorothioate oligoribonucleotides (see Chapter 4)

An individual phosphodiester bond can be substituted for a phosphorothioate bond (racemic mixture of R_p and S_p isomers) by interrupting the synthesizer programme at the appropriate cycle just before the step of addition of the oxidation reagent and by carrying out manual sulphurization (*Protocol 14*).

Protocol 14. Manual sulphurization of oligoribonucleotides

1. Remove the column from the synthesizer.
2. Connect a syringe filled with a Luer adaptor, containing 0.4 g elemental sulphur (Aldrich gold label) in carbon disulphide/2,6-lutidine (6 ml).
3. Inject 1 ml of the sulphurization solution every hour until all the solution has passed through.

Protocol 14. *Continued*

4. Wash the column with carbon disulphide/2,6-lutidine (10 ml) and then with aceonitrile (20 ml).
5. Reconnect the column to the synthesizer and continue the assembly cycle as though starting a new cycle (i.e. acidic deprotection step).

After deprotection of the oligonucleotide in the normal way, ion exchange HPLC should show a major peak the elution position of which should be slightly retarded compared with the natural oligoribonucleotide. In favourable cases, the R_p and S_p isomers may be resolved by subsequent reversed phase HPLC (21).

We do not recommend carrying out the sulphurization on the synthesizer since elemental sulphur tends to precipitate in the delivery line thus clogging it. A 1 hour sulphurization using the new reagent tetraethylthiuram disulphide (Lancaster Synthesis, 500 mg) in acetonitrile (5 ml) has recently obviated the clogging problem (21).

8. Future developments

The currently available range of commercial ribo amidites and support-bound nucleosides is adequate for the synthesis of medium length oligoribonucleotides (up to perhaps about 30–40 residues). It is likely that for the synthesis of longer oligoribonucleotides significant improvements in chemistry will be needed. The use of base-protecting groups removable under milder ammoniacal conditions (18, 19) will be helpful and phenyoxyacetyl-protected A and G ribo amidites have very recently become available (American Bionetics and Glen Research). However, better coupling yields will be essential and this can only come through development of more active ribo amidites or more active activating agents. In order to avoid possible cleavage by ribonucleases during purification, it would also be very useful if oligoribonucleotides could be purified at the 2′-protected stage, as is possible using 2′-Mthp protected oligoribonucleotides (7). It remains to be seen whether procedures can be found for either 2′-TBDMS (described here) or 2′-Fpmp (13) containing oligoribonucleotides to be routinely purified after assembly.

One exciting prospect which should now become possible is the ability to incorporate modifications to either phosphate, sugar, or base or combinations of modifications into specific locations within oligoribonucleotides for use for example in the study of RNA–protein interactions or of self-cleaving RNA domains and ribozymes.

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3

2′-O-Methyloligoribonucleotides: synthesis and applications

B. S. SPROAT and A. I. LAMOND

1. Introduction

2′-O-Methyloligoribonucleotides (1–7) are proving to be useful reagents for a variety of biological experiments. Their usefulness stems from the following properties:

- a 2′-O-methyloligoribonucleotide–RNA duplex is thermally more stable than the corresponding oligodeoxyribonucleotide–RNA one (2) and
- the former duplex is not a substrate for RNase H (6).

This enzyme specifically cleaves RNA in RNA–DNA heteroduplexes (8). In addition, 2′-O-methyloligoribonucleotides are chemically more stable than either oligodeoxyribonucleotides or oligoribonucleotides and moreover are totally resistant to degradation by either RNA- or DNA-specific nucleases (7).

The first part of this chapter describes in detail the synthesis of appropriately protected 2′-O-methylribonucleoside-3′-O-phosphoramidites (9) and ancillary reagents required for solid phase assembly of 2′-O-methyloligoribonucleotides. This part also includes procedures for synthesis, biotinylation (10), deprotection, and purification of the polymers. The methods described here have been limited to the most commonly used solid phase synthesis method, the so-called phosphite triester method (11–13). However, with minor changes to the protocols, H-phosphonate or phosphodiester building blocks can be easily prepared.

The second part of the chapter describes some of the applications of 2′-O-methyloligoribonucleotides, in particular their usage for affinity chromatography of RNA–protein complexes. This has recently proved important for *in vitro* studies of RNA processing (14–16). (See also Chapter 10 for oligodeoxynucleotides for affinity chromatography and Chapter 11 for oligodeoxynucleotides, with reporter groups attached to the base.)